Novel Dual-Fluorescence Cell-Based ELISAs for Analysis of Intracellular Proteins or Phosphorylation of Signaling Molecules

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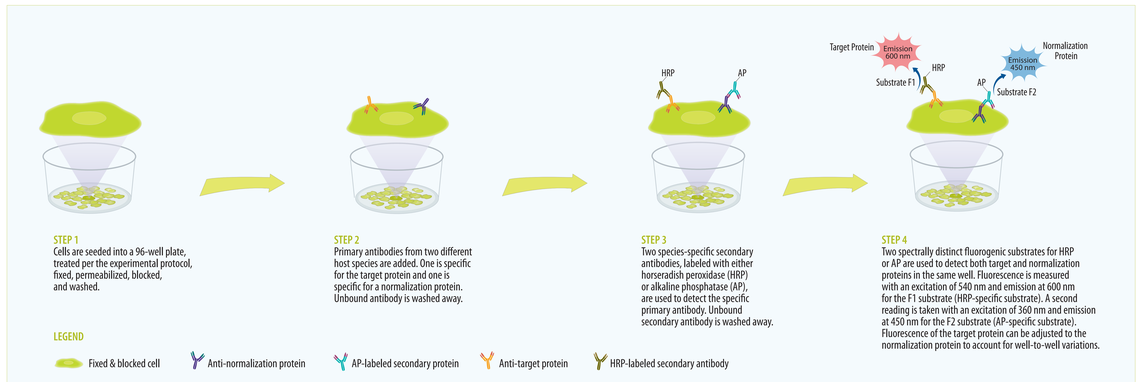
ABSTRACT

We have developed a fluorogenic cell-based ELISA that does not require lysate preparation. In using this format, two target cellular proteins can be analyzed simultaneously in the same well, thus minimizing assay variability. Furthermore, this assay is more rapid than Western blotting and is suitable for high-throughout applications. We have used this technique to assess unique protein phosphorylations, and to evaluate the effects of stimulators and inhibitors on cultured cells. Because of the assay's dual detection capabilities, the phosphorylation signal can be normalized to the signal from the total protein, thus providing a more accurate measurement of any given change. Cells are grown in 96-well plates and stimulated with ligands and/or incubated with inhibitors. They are then fixed and permeabilized in the wells. This is followed by incubation with two primary antibodies derived from different species: a phospho-specific antibody and a normalization antibody that recognizes the total protein regardless of its phosphorylation status. Species-specific secondary antibodies labeled with horseradish peroxidase (HRP) and alkaline phosphatase (AP), and spectrally distinct fluorogenic substrates for each enzyme are used for detection. The fluorescence of phosphorylated protein is normalized to that of the total protein in each well to correct for well-to-well variations. This two-wavelength cell-based assay was used to assess phosphorylation of ERK1/2, Akt, p38 MAPK, EGF R, STAT3, and total protein levels of HIF-1α induced by various stimuli and the effects of kinase inhibitors. The results correlate closely with those measured by classical methodologies, such as Western blotting.

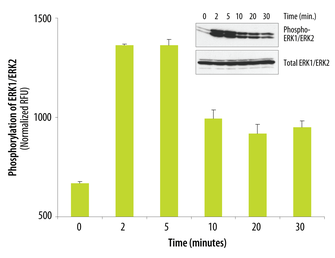
MATERIALS & METHODS

Product	Catalog #
Human/Mouse/Rat Phospho-Akt (S473) Cell-Based ELISA	KCB887
Human Phospho-EGF R (Y1068) Cell-Based ELISA	KCB1095
Human/Mouse/Rat Phospho-ERK1/ERK2 (T202/Y204) Cell-Based ELISA	KCB1018
Human/Mouse/Rat Phospho-p38 MAP Kinase (T180/Y182) Cell-Based ELISA	KCB869
Human/Mouse Phospho-STAT3 (Y705) Cell-Based ELISA	KCB4607
Human/Mouse Total HIF-1α Cell-Based ELISA	KCB1935

ASSAY SCHEMATIC



1A.



1B.

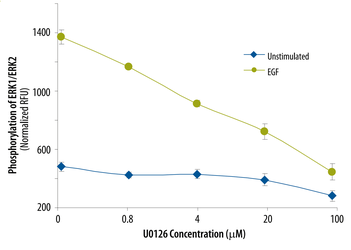
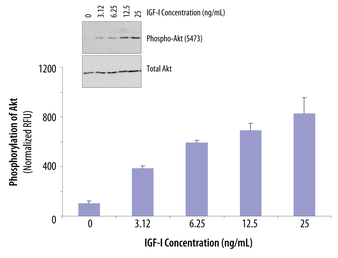


Figure 1: Measurement of ERK1/ERK2 phosphorylation in A431 cells using the Phospho-ERK1/ERK2 (T202/Y204) Cell-Based ELISA Kit (Catalog # KCB1018). A431 human epidermoid carcinoma cells were seeded at approximately 1.0 x 10^4 cells per well in 96-well plates 16 hours before treatment. Cells were treated with 100 ng/mL of recombinant human (rh) EGF (R&D Systems, Catalog. # 236-EG) for the indicated times **(A)**, or were pretreated for one hour with the indicated concentrations of the MEK inhibitor U0126 and then incubated with no additions or with 1 ng/mL rhEGF for 30 minutes **(B)**. After fixation of cells in the wells, phosphorylation of ERK1/ERK2 was determined and normalized to total ERK1/ERK2 in the same well. Values represent mean ± range of duplicate determinations. Western blot analysis of ERK1/ERK2 phosphorylation and total ERK1/ERK2 using the antibodies supplied in this cell-based ELISA kit is also shown (inset of Fig. 1A).

2A.



2B.

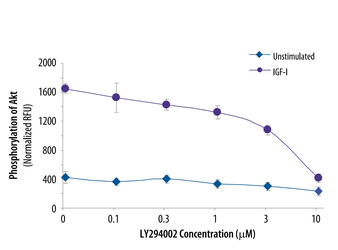
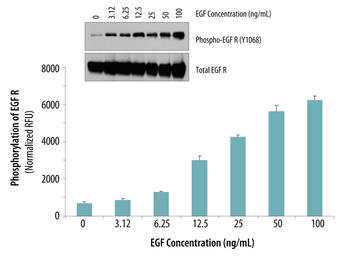


Figure 2: Measurement of Akt phosphorylation in MCF-7 cells using the Phospho-Akt (S473) Cell-Based ELISA Kit (Catalog # KCB887). MCF-7 human breast adenocarcinoma cells were seeded at approximately 1.5 x 10^4 cells per well in 96-well plates 16 hours before treatment. Cells were treated with increasing amounts of recombinant human (rh) IGF-I (R&D Systems, Catalog # 291-G1) for 20 minutes **(A)**, or were pretreated for 10 minutes with the indicated concentrations of the PI 3-kinase inhibitor LY294002 and then incubated with no additions or with 25 ng/mL rhIGF-I for 20 minutes **(B)**. After fixation of cells in the wells, phosphorylation of Akt on S473 was determined and normalized to total Akt in the same well. Values represent mean ± range of duplicate determinations. Western blot analysis of Akt phosphorylation and total Akt using the antibodies supplied in this cell-based ELISA kit is also shown (inset of Fig. 2A).

3A.



3B.

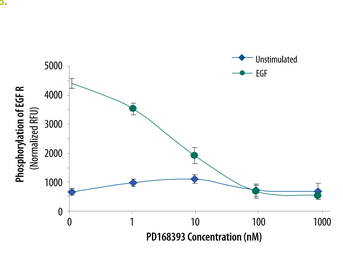
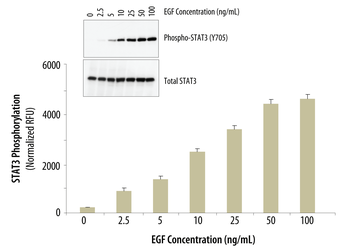


Figure 3: Measurement of EGF R phosphorylation in A431 cells using the Phospho-EGF R (Y1068) Cell-Based ELISA Kit (Catalog # KCB1095). A431 human epidermoid carcinoma cells were seeded at approximately 1.0 x 10^4 cells per well in 96-well plates 16 hours before treatment. Cells were treated with increasing amounts of recombinant human (rh) EGF (R&D Systems, Catalog # 236-EG) for 5 minutes **(A)**, or were pretreated for 30 minutes with the indicated concentrations of the EGF R tyrosine kinase inhibitor PD168393 and then incubated with no additions or with 50 ng/mL rhEGF for 5 minutes **(B)**. After fixation of cells in the wells, phosphorylation of EGF R on Y1068 was determined and normalized to total EGF R in the same well. Values represent mean ± range of duplicate determinations. Western blot analysis of EGF R phosphorylation and total EGF R using the antibodies supplied in this cell-based ELISA kit is also shown (inset of Fig. 3A).

4A.



4B.

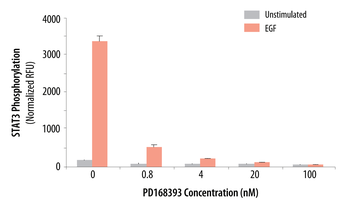


Figure 4: Measurement of STAT3 phosphorylation in A431 cells using the Phospho-STAT3 (Y705) Cell-Based ELISA Kit (Catalog # KCB4607). A431 human epidermoid carcinoma cells were seeded at approximately 1.5 x 10^4 cells per well in 96-well plates 16 hours before treatment. Cells were treated with increasing amounts of recombinant human (rh) EGF (R&D Systems, Catalog # 236-EG) for 10 minutes **(A)**, or were pretreated for 60 minutes with the indicated concentrations of the EGF R tyrosine kinase inhibitor PD168393 and then incubated with no additions or with 50 ng/mL rhEGF for 10 minutes **(B)**. After fixation of cells in the wells, phosphorylation of STAT3 on Y705 was determined and normalized to total STAT3 in the same well. Values represent mean ± range of duplicate determinations. Western blot analysis of STAT3 phosphorylation and total STAT3 using the antibodies supplied in this cell-based ELISA kit is also shown (inset of Fig. 4A).

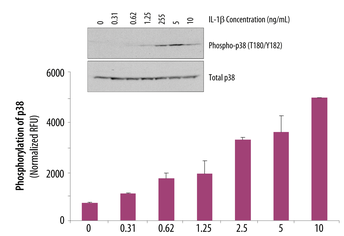


Figure 5: Measurement of p38 phosphorylation in HepG2 cells using the Phospho-p38 MAP Kinase (T180/Y182) Cell-Based ELISA Kit (Catalog # KCB869). HepG2 human hepatocellular carcinoma cells were seeded at approximately 1.5 x 10^4 cells per well in 96-well plates 16 hours before treatment. Cells were treated with increasing amounts of recombinant human (rh) IL-1β (R&D Systems, Catalog # 201-LB) for 25 minutes. After fixation of cells in the wells, phosphorylation of p38 on T180/Y182 was determined and normalized to total p38 in the same well. Values represent mean ± range of duplicate determinations. Western blot analysis of p38 phosphorylation and total p38 using the antibodies supplied in this cell-based ELISA kit is also shown (inset).

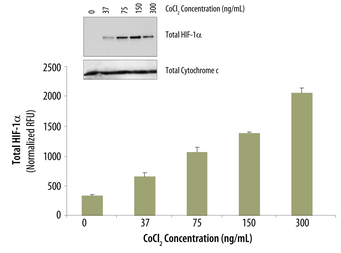


Figure 6: Measurement of HIF-1α protein levels in MCF-7 cells using the Total HIF-1α Cell-Based ELISA Kit (Catalog # KCB1935). MCF-7 human breast adenocarcinoma cells were seeded at approximately 1.5 x 10^4 cells per well in 96-well plates 16 hours before treatment. Cells were treated with the indicated amounts of $CoCl_2$, a hypoxia mimetic, for 8 hours. After fixation of cells in the wells, HIF-1α levels were determined and normalized to total cytochrome (a housekeeping protein) in the same well. Values represent mean ± range of duplicate determinations. Western blot analysis of total HIF-1α and total cytochrome c using the antibodies supplied in this cell-based ELISA kit is also shown (inset).

SUMMARY

Although *in vitro* biochemical kinase assays are routinely used for drug screening, they cannot replicate the intracellular environment. Cell-based assays measuring unique events within specific signaling pathways may reflect the intracellular environment more closely. Our assay is the first dual-fluorescence cell-based ELISA to measure two intracellular proteins simultaneously within the same well. Using this assay format, two target proteins can be analyzed or, alternatively, one protein can be used to normalize for well-to-well variations in cell number. Two types of assays have been developed. The first type of assay uses primary antibodies against phospho- and total proteins when assessing protein phosphorylation status of signaling molecules. In the second type of assay, we employ antibodies against the protein of interest and a second housekeeping protein for the measurement of total protein levels of the target molecule. Both types of assays enable a reliable measurement of changes in cellular proteins in whole cells by ratiometric analysis of two wavelengths derived from the same well to minimize variability. Assay detection using species-specific HRP- or AP-conjugated secondary antibodies in combination with spectrally distinct fluorogenic substrates allows for measurement with a standard fluorescence plate reader. Therefore, there is no need for specialized and expensive equipment. The effects of well-studied kinase inhibitors on their respective kinases were evaluated using this cell-based ELISA, and the results were similar to those previously reported using different techniques.

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